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AUTHORITY

**Biological Defense Research Lab ltr dtd 28  
Sep 1971**

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AD 476512

TECHNICAL MANUSCRIPT 272

COCCIDIOIDIN SKIN TESTS  
AND SEROLOGIC REACTIONS  
IN IMMUNIZED AND INFECTED MONKEYS

Edwin P. Lowe  
James T. Sinski  
Milton Huppert  
John G. Ray, Jr.

DECEMBER 1965

UNITED STATES ARMY  
BIOLOGICAL LABORATORIES  
FORT DETRICK

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Special Operations Division  
DIRECTORATE OF DEVELOPMENT  
and  
Medical Investigation Division  
DIRECTORATE OF MEDICAL RESEARCH

Project 1C522301A059

December 1965

In conducting the research reported here, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

### FOREWORD

Dr. Milton Huppert is Chief of Mycology Research Laboratory, Veterans Administration Hospital, San Fernando, California.

### ACKNOWLEDGMENT

The authors wish to acknowledge with gratitude the assistance of Captains Peter J. Soto, Jr., and Vilas E. Misner, U.S. Army, who performed the histopathologic studies connected with the experiment.

### ABSTRACT

The coccidioidin skin test and serologic reactions in immunized and nonimmunized monkeys are described. Experimental vaccines included killed intact cells and cell fraction antigens. Serologic tests included tube precipitin, two different agar gel immunodiffusion techniques, and two similar complement fixation (CF) tests.

Skin and serologic activity was determined also in animals after aerosol infection. Both classes of reactions were greater in infected than in noninfected animals. However, the serologic response of monkeys to the disease differed in several aspects from that reported for man. Tube precipitin reaction was sporadic and where recorded tended to persist. Complement fixation reactions in infected monkeys were most nearly similar to those reported for man except that the monkey response generally was greater. Positive CF and agar gel immunodiffusion reactions were recorded in some animals early in the disease. Both types of agar gel immunodiffusion reactions paralleled the CF reactions, and they may all be tests for the same antibody. The value of these tests in immunologic studies *in vivo* with Coccidioides immitis is discussed.

## I. INTRODUCTION

The animal protection test generally has been used to determine the protective efficacy of experimental vaccines against coccidioidomycosis.<sup>1-3</sup> The coccidioidin skin test and certain serologic reactions have proved useful in diagnosis of human and animal coccidioidomycosis.<sup>4-6</sup> Therefore, it seemed desirable to investigate their value in detecting animal response to inoculation with experimental antigens. If they could be so used, much time could be saved and perhaps more precise information on antigen potency be obtained. Accordingly, arrangements were made to include skin and serological tests in a contemplated study of the protective response of three experimental vaccines in monkeys. The serological tests were to be carried out by two independent laboratories, one of which was concerned chiefly with human coccidioidal serology. This report deals with the results of that study.

## II. MATERIALS AND METHODS

### A. ANIMALS

Twenty Macaca mulatta monkeys, of both sexes, each weighing approximately 3 kg were used in the investigation. They were divided into four groups of five animals each. Each of first three groups was inoculated with a different nonviable vaccine, the fourth was reserved as a nonimmunized control group.

### B. ANTIGENS

Three nonviable C. immitis vaccines were used. The first was a suspension of C. immitis, strain M11, spherules grown in liquid culture according to the method of Converse.<sup>7,8</sup> Their viability was destroyed by suspension in 0.5% formalin and then resuspension in physiological saline on a weight basis. The second vaccine was an acetone-pyridine extract of C. immitis, strain Silveira, arthrospores that was combined with pertussis vaccine as described elsewhere.<sup>9</sup> In the third vaccine, killed arthrospores of the same strain were subjected to enzyme digestion in the hope that the treatment would enhance antigen release. This vaccine was prepared by Drs. N.F. Conant and H.F. Hardin, Duke University.

#### C. VACCINE ADMINISTRATION

The total immunizing dose with all test antigens was 24 mg (calculated dry weight) and was administered in four subcutaneous injections. The regimen consisted of two 8-mg injections of vaccine at 0 and 2 weeks and two 4-mg injections at approximately 6 and 14 weeks.

#### D. SKIN TESTS

All monkeys were skin-test negative to old tuberculin. They were skin-tested with undiluted coccidioidin before vaccine administration, during immunization, and after injection. The coccidioidin was prepared in this laboratory by a method described elsewhere<sup>9</sup> and was comparable in strength to a standard lot supplied by Dr. C.E. Smith.\* The coccidioidin was administered by injecting 0.1 ml into the upper eyelid. Both eyelids were used to avoid successive tests in the same site. Readings were made at 24 and 48 hours, and both erythema and induration were considered in the readings.

#### E. SEROLOGY

Blood samples were collected from all animals at intervals during the study to determine their serological response during immunization and after challenge with live organisms. All collected blood samples were coded and submitted to our two separate laboratories for analysis. The tests used were the standard diagnostic tube precipitin (TP) and the complement fixation (CF) tests,<sup>10,11</sup> and the agar gel immunodiffusion (AGID) of Huppert and Bailey<sup>12</sup> and agar gel precipitin inhibition (AGPI) test of Ray and Kadull.<sup>13</sup> Sera were not pretreated for the agar gel precipitin tests, but were pretreated with complement in the CF determinations to eliminate anticomplementary activity found in animal sera.<sup>10</sup>

#### F. RESPIRATORY EXPOSURE

Approximately one month after antigen administration was completed, animals were exposed to a static aerosol of dry C. immitis, strain Silveira, by a method previously described.<sup>14</sup> Exposure time was varied to secure as nearly as possible a standard inhaled dose that would cause clinical but not lethal disease. Inhaled doses were calculated from animal breathing rates and viable particle counts of air samples collected during exposure. The average challenge dose was 20 arthrospores (range 11 to 58).

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\* School of Public Health, University of California, Berkeley.

#### G. POST-CHALLENGE ANIMAL TREATMENT, EXPERIMENTAL PROTOCOL

After challenge, animals were housed in individual cages in a gastight, air-conditioned cabinet system. The experiment was terminated approximately 13 weeks after animal challenge. All animals were sacrificed by Nembutal injection and pathologic examinations were made of tissue collected at autopsy.

The time sequence for skin tests, antigen administration, blood sample collection, and animal challenge and sacrifice is summarized in Table 1.

TABLE 1. TIME SEQUENCE FOR MONKEY IMMUNIZATION EXPERIMENT

Days	Skin Test Number	Blood Sample Number	Immunizing Dose Number	Challenge	Sacrifice
Control	1	1			
0			1		
13	2		2		
27	3				
34		2			
42			3		
62	4				
103	5	3	4		
124	6				X
135					X
142		4			
149		5			
193	7	6			
215		7			
220					X

### III. RESULTS AND DISCUSSION

#### A. INFECTION IN IMMUNIZED AND NONIMMUNIZED MONKEYS

The comparative disease status and postchallenge serologic reactions of immunized and nonimmunized animals are shown in Table 2.

The animals appeared healthy at sacrifice, but pathologic examination disclosed infection in all. The infections were graded from severe to mild on the basis of extent of pulmonary involvement, nature of lesions, and evidence of dissemination. Except that the disease tended to be somewhat more severe in the nonimmunized animal group, there were no marked differences between infections in the several groups. Nonviable *C. immitis* vaccines generally protected against death but did not prevent some degree of infection.<sup>2,15,16</sup> It had been hoped that with use of sublethal challenge doses one or more test antigens would provide complete protection.

#### B. INTRADERMAL TESTS

Coccidioidin and tuberculin skin tests were negative in all animals until after challenge. Slight erythema and/or induration of less than 5 by 5 mm lasting 24 to 48 hours were observed sporadically in all vaccinated animals. This type of reaction was noted most frequently in animals receiving the killed spherule antigen but were not consistent in their occurrence in any group. After challenge, coccidioidin skin test reactions in both immunized and control animals were definitely positive at 24- and 48-hour readings.

#### C. SEROLOGY

Serologic reactions during immunization were all negative. Serologic reactions were also negative at one week postchallenge, but thereafter reactions were positive with sera from all test groups (Table 2).

The agar gel precipitin and complement fixation tests may have some ancillary potential in evaluating the protective efficacy of experimental vaccines in animal protection tests. Positive reactions were recorded at 2 weeks postchallenge in some animals immunized with the killed spherule preparation, the antigen that generally affords the greatest protection against death in animal protection tests. The AGPI test appeared the most sensitive, as indicated by titer and number of animal sera reacting positively (Table 2). Only the serum that gave the highest titer at 2 weeks in the AGPI test reacted positively in the CF test and afforded a marginally positive reaction in the AGID test. Although no marked differences were noted, CF titers tended to be lower in the immunized groups of animals than in the nonimmunized controls. They were lowest in animals inoculated with killed spherule and the cell fraction-pertussis vaccine combination. CF reactions were uniformly negative in one animal and titers were low in a second animal in each group. The slowest serologic response

TABLE 2. COMPARISON OF POSTCHALLENGE SEROLOGIC REACTIONS IN IMMUNIZED AND NONIMMUNIZED MONKEYS.

Immunizing Antigen	Monkey	Disease Status <sup>a</sup>	Tube Precipitin Test						AGID Test						Complement Fixation Test						
			2 wk		8 wk		12 wk		2 wk		8 wk		12 wk		2 wk		8 wk		12 wk		
			+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	
Intact Spherule	K-21	3+	0	0	0	0	+	-	+	-	+	-	+	-	32b/	512	1024	8	0	2	20
	K-43	2+	0	NS <sup>b</sup> /	+	0	+	-	+	-	+	-	+	-	4	128	1024	0	8	8	0
	K-11	1+	0	0	0	0	0	+	0	0	0	0	0	0	4	128	256	0	8	4	NS
	K-20	2+	0	0	0	0	0	+	0	0	0	0	0	0	0	128	1024	0	0	0	NS
	P-17	1+	0	0	0	0	0	+	0	0	0	0	0	0	0	32	256	0	AC <sup>d</sup> /	0	0
Combined Cell Fraction	S-49	2+	0	0	+	0	0	+	0	0	+	0	0	0	0	8	4	0	0	0	0
	K-45	3+	0	0	+	0	+	0	+	0	+	0	0	0	0	512	1024	0	8	512	AC
	P-12	2+	0	0	0	0	+	0	0	0	0	0	0	0	0	256	1024	0	16	256	0
	P-14	1+	0	0	+	0	+	0	0	0	0	0	0	0	0	128	1024	0	4	32	0
	P-15	2+	0	0	0	0	0	0	0	0	0	0	0	0	0	16	8	0	0	0	10
Cell Enzyme Digest	P-27	1+	0	0	0	0	0	0	+	0	+	0	0	0	0	64	256	0	4	128	0
	K-3	1+	0	+	0	0	0	0	0	0	0	0	0	0	0	32	64	0	4	64	0
	P-3	1+	0	0	+	0	0	+	0	0	0	0	0	0	0	128	256	0	8	256	0
	T-108	2+	0	0	0	0	0	0	0	0	0	0	0	0	0	512	1024	0	16	512	0
	P-1	2+	0	+	0	+	0	+	0	0	+	0	0	0	0	128	256	0	16	128	AC
Non-Immunized	X-17	2+	0	0	0	0	0	0	+	0	+	0	0	0	0	128	1024	0	8	128	0
	X-58	3+	0	0	0	0	0	0	0	0	0	0	0	0	0	512	1024	0	32	512	0
	X-67	1+	0	+	0	0	+	0	0	0	0	0	0	0	0	64	64	0	8	32	0
	X-69	3+	0	+	0	0	+	0	0	0	0	0	0	0	0	512	1024	0	32	1024	0
	S-65	3+	0	0	0	0	0	0	+	0	0	0	0	0	0	256	256	0	4	128	0

a. Relative extent of infections (1+, mild; 2+, moderate; 3+, severe).

b. Reciprocal of tube dilution.

c. No sample.

d. Anticomplementary.

was that with the tube precipitin test, in which positive reactions were sporadic in all animal groups. No consistent relationship was observed between serologic reactions and severity of disease as determined by pathologic study. Low-titered or negative serologic reactions were recorded in some animals with severe infections as well as relatively high-titered reactions in some animals with moderate to mild disease.

There was also relatively good correlation between results of the qualitative AGID and the quantitative AGPI test except that the latter appeared somewhat more sensitive. Figure 1 shows the relationship in results of CF tests conducted independently by the serological laboratories at Veterans Administration Hospital, San Fernando, California and Fort Detrick, Frederick, Maryland. Previous studies of coccidioidomycosis in monkeys indicated that CF reactions tend to persist, even in relatively mild disease.<sup>3,14,15</sup> Both laboratories reported approximately the same pattern of CF titer response among immunized and nonimmunized animal groups, except that titer levels were generally higher in the Fort Detrick tests. The lowest titers occurred in animals inoculated with the spherule suspension and the highest in the nonimmunized control group at both laboratories.

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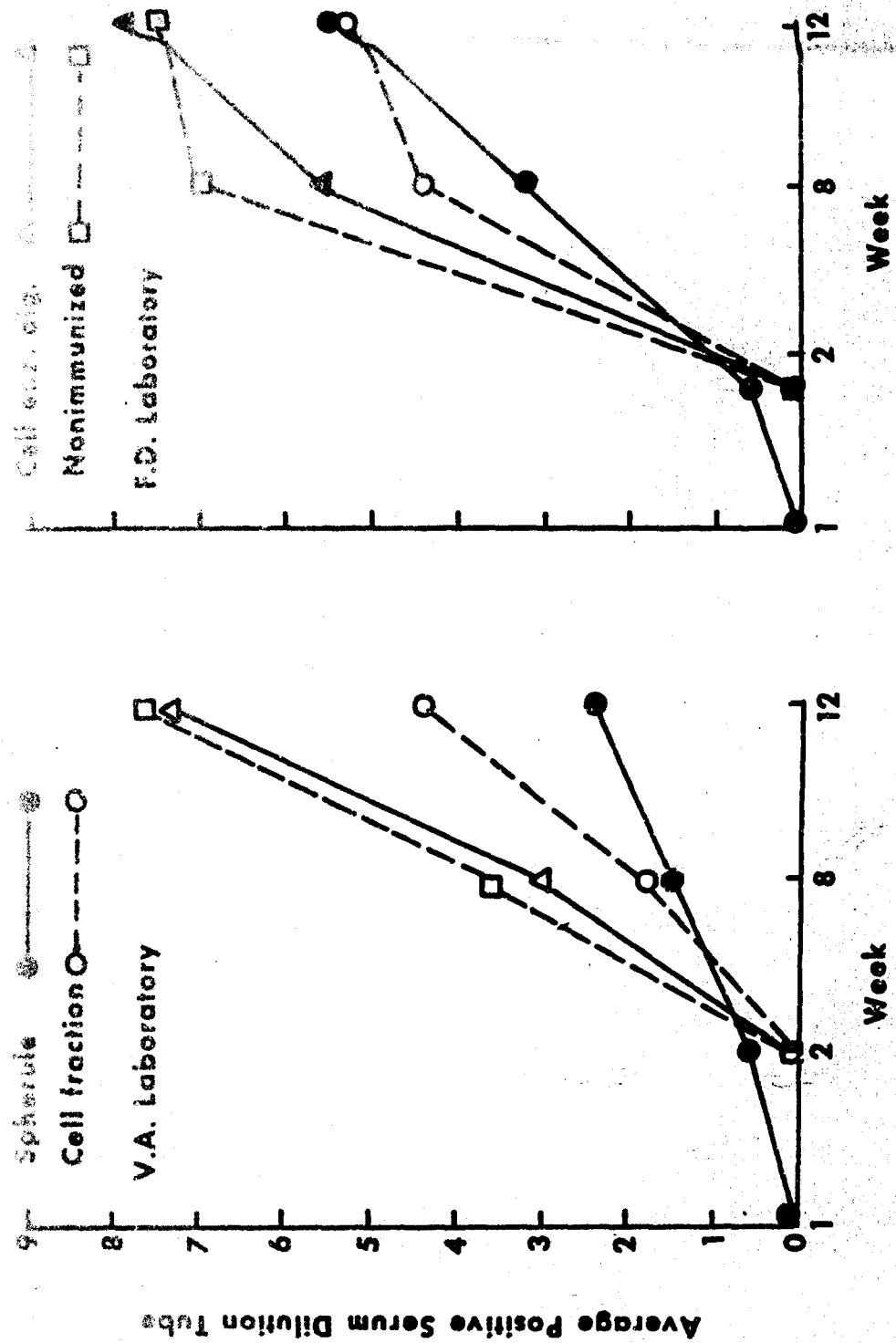


Figure 1. Comparison of Postchallenge Complement Fixation Reactions in Immunized and Nonimmunized Monkeys. Tests at the Veterans Administration laboratory were made by Dr. Huppert; those at Fort Detrick were made by Dr. Ray.

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Unclassified  
Security Classification

DOCUMENT CONTROL DATA - R&D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author)

U.S. Army Biological Laboratories  
Fort Detrick, Frederick, Maryland, 21701

2a. REPORT SECURITY CLASSIFICATION

Unclassified

2b. GROUP

3. REPORT TITLE

COCCIDIOIDIN SKIN TESTS AND SEROLOGIC REACTIONS IN IMMUNIZED AND INFECTED MONKEYS

4. DESCRIPTIVE NOTES (Type of report and inclusive dates)

5. AUTHOR(S) (Last name, first name, initial)

Lowe, Edwin P. Ray, John G., Jr.  
Sinski, James T.  
Huppert, Milton, NMT

6. REPORT DATE

December 1965

7a. TOTAL NO. OF PAGES

16

7b. NO. OF REPS.

16

8a. CONTRACT OR GRANT NO.

8b. ORIGINATOR'S REPORT NUMBER(S)

b. PROJECT NO. 1C522301A059

Technical Manuscript 272

c.

9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)

d.

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11. SUPPLEMENTARY NOTES

12. SPONSORING MILITARY ACTIVITY

U.S. Army Biological Laboratories  
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Skin and serologic activity was determined also in animals after aerosol infection. Both classes of reactions were greater in infected than in noninfected animals. However, the serologic response of monkeys to the disease differed in several aspects from that reported for man. Tube precipitin reaction was sporadic and where recorded tended to persist. Complement fixation reactions in infected monkeys were most nearly similar to those reported for man except that the monkey response generally was greater. Positive CF and agar gel immunodiffusion reactions were recorded in some animals early in the disease. Both types of agar gel immunodiffusion reactions paralleled the CF reactions, and they may all be tests for the same antibody. The value of these tests in immunologic studies *in vivo* with Coccidioides immitis is discussed.